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Synthesis and Application of the Doxaz-MARCKS Prodrug

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Abstract

Chemotherapeutic prodrugs have demonstrated success in killing cancer cells; however they also pose harm to healthy cells. The objective of this project was to synthesize an improved prodrug that is selectively activated only in the cancer microenvironment, thus minimally affecting healthy tissue. A photolabile linker was hypothesized to achieve said objective. The Yin group discovered that the MARCKS-ED peptide can localize to the exosome via curvature sensing and electrostatic interactions. In addition, the Koch group determined that Doxazolidine (Doxaz) is more cytotoxic than its clinical drug precursor. Also, no cancer cell lines have shown resistance to this anthracycline. Based on these studies, we decided to synthesize a prodrug containing a photolabile linker, MARCKS-ED peptide, and the Doxaz anthracycline. We hypothesized that a specific sequence of synthesis and purification steps would generate the prodrug. We carried out steps and characterized the products using NMR, HPLC and ESI-MS. The results confirmed that the desired product was produced. It was then predicted that application of UV light will activate the prodrug and cause cell death. IC_{50} values determined that application of UV light after prodrug incubation significantly decreased cell viability in comparison to cells retained in the dark. Further experiments are needed to verify the mechanism of the prodrug and ensure its efficacy in-vivo. Nonetheless, these results suggest that Doxaz-MARCKS will serve as a pragmatic chemotherapeutic.

Introduction

Cancer has been a lasting epidemic. An estimated 14 million people are diagnosed globally every year and approximately 8 million loose the battle.[1] Chemotherapeutics are a common form of treatment for the pathology; in fact, more than a hundred are used today.[2] Chemotherapy has demonstrated success in killing cancer cells; however, it still poses harm to healthy cells due to its limited ability to distinguish between the two.

Further work is needed to create a compound that only targets cancer, yet minimally affects healthy tissue. The application of prodrugs has implemented an idea of a selective marker that will activate the cytotoxic agent only in the cancer microenvironment, while leaving healthy cells unharmed. Since some cancers may overexpress certain receptors and proteases, previously used prodrugs relied on enzymatically cleavable linkers. The PPD Prodrug is one example.[3] It implements an ester linker that serves as a substrate for the CES2 carboxylesterase, an enzyme overexpressed in certain cancer cell lines.

However, there is a dilemma associated with enzymatically labile linkers. Healthy cells may still express the enzyme to some extent, while some cancer cell lines may not overexpress it as anticipated. Indeed, PPD demonstrated success in killing cancer cells, however, it caused hepatotoxicity because CES2 is also highly expressed in the liver.[3] To avoid the risk associated with proteolytic markers, this project proposed to implement a photolabile linker that dissociates upon irradiation with UV light, hence activating the prodrug. The chosen linker for this project is shown in **Figure 1a**; it dissociates via a photochemical mechanism with radical intermediates (**Figure 1b**).[4]

Figure 1: a) Photolabile linker that will be utilized for this project and b) its UV-induced dissociation mechanism.

For increased efficiency, it is best if the prodrug is also localized to the cancer. This project focused on doing so via MARKCS-exosome targeting. MARCKS is an endogenous peptide that binds phosphatidylserine (PS) lipids on the inner lipid leaflet of cells.[4] Its effector domain (ED), composed of 25 amino acids, is responsible for this. The Yin group determined that MARCKS is able to localize to exosomes due to its abundance of K and P residues in the ED sequence.[5-6]

Exosomes are microvesicles that are produced by cells for autocrine and paracrine signaling.[7] Their diameter ranges from 30-130 nm, resulting in high membrane curvature and exposure of hydrophobic hydrocarbons.[5-6,8] In addition, the outer leaflet of these vesicles also harbors negatively charged PS lipids.[7] MARCKS-ED is able to localize to the exosome by electrostatic interactions between the anionic PS and cationic K residues, as well as by F residues, which insert between the lipids of the highly curved exosomal membrane (**Figure 2**).[5-6]

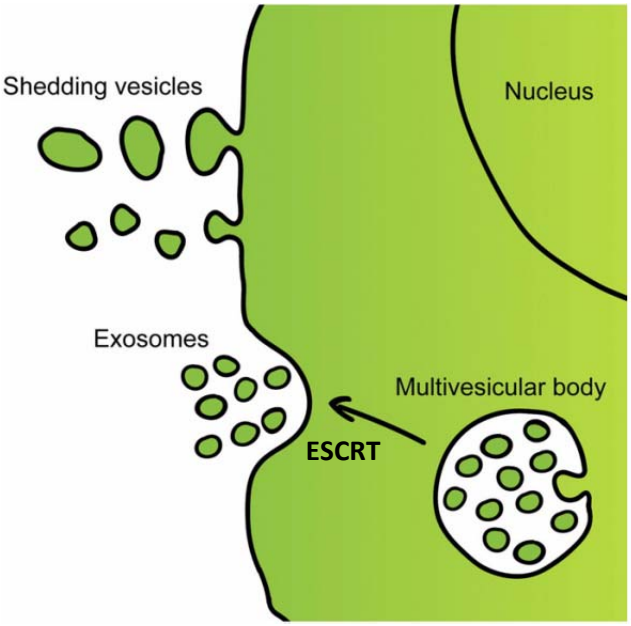


Figure 2: Exosomes interact with cells either via the ESCRT pathway or membrane fusion. Figure courtesy of Dommelen, S. M., Vader, P., Lakhal, S.; Kooijmans, S. A. A., van Solinge, W. W., Wood, M. J. and Schiffelers, R. M.[10]

There are advantages to using the exosome delivery system instead of the liposome or the nanoparticle. Exosomes are endogenous to the body, thus they will not produce an immunogenic response that a nanoparticle may trigger.[9] Its natural characteristics also allow them to travel long distances via circulation.[7,9] In addition, they possess receptors, such as CD9 and CD63, which allow them to either fuse with the cell membrane or be endocytosed via an ESCRT pathway (**Figure 2**).[7,10] Lastly, cancer cell lines generally produce an excessive amount of exosomes in comparison to that of healthy cells and may even use these microvesicles for metastasis.[7,10] All these features make the exosome a practical drug delivery system for a chemotherapeutic.

Lastly, the most significant part of a chemotherapeutic is the cytotoxic agent. For our prodrug, we chose Doxazolidine (Doxaz), an anthracycline derived from Doxorubicin (Dox).[11] Addition of formaldehyde to Dox covalently links its 3'-amino and 4'-hydroxyl to form an oxazolidine ring of Doxaz. The structure of Dox and Doxaz are displayed in **Figure 3**, respectively.

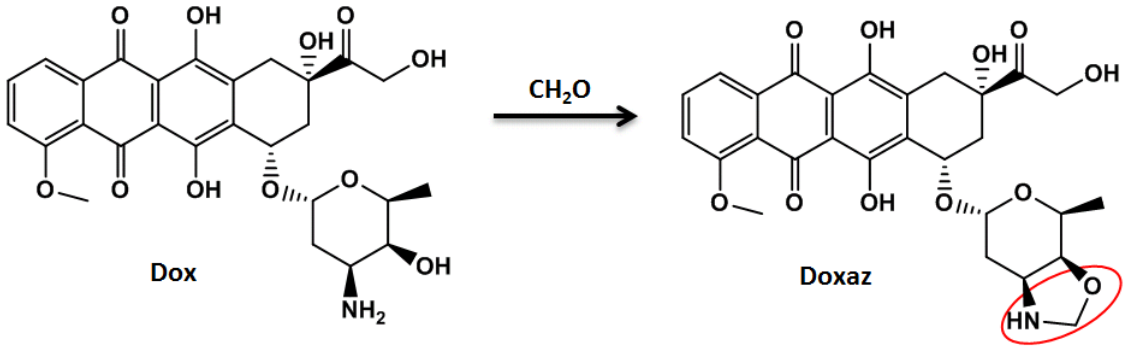


Figure 3: Structures of Dox and Doxaz only differ in the methylene carbon, which forms on oxazolidine ring on the latter (circled in red).

The original Dox was discovered from the Streptomyces soil bacteria in 1969 and has been renowned for its anti-tumor effects.[12-13] It wasn't long until it was administered as an anti-cancer drug; yet later it was discovered to cause severe cardiomyopathy in its patients, leading to congestive heart failure years after administration.[14] Since, the Koch group has been working with the Doxaz compound, which they revealed to have many advantages in relation to its precursor.[11]

For starters, Doxaz does not exhibit selective toxicity towards cardiomyocytes because it exhibits a different mechanism of action.[11,15] Unlike Dox, which is dependent on Topoisomerase II, Doxaz is enzyme independent. It directly crosslinks DNA at 5'-GC-3' regions, a lesion that triggers apoptosis (**Figure 4**).[11] Also, the IC_{50} values of growth inhibition by Doxaz were measured by the National Cancer Institute across 60 cancer cell lines, all of which were one to four magnitudes lower than those for Dox.[11, 16] Some cell lines examined possessed the multidrug resistant (MDR) phenotype and some overexpressed P170 glycoprotein efflux pump (P170GP), which exports hydrophobic molecules with cationic charges.[17] Dox is cationic under physiological pH, and has been reported to be a substrate for the pump. Consequently, cell lines that express MDR are resistant to it. Doxaz is cytotoxic to MDR cells likely because it is unprotonated at physiological pH of 7.4.[11]

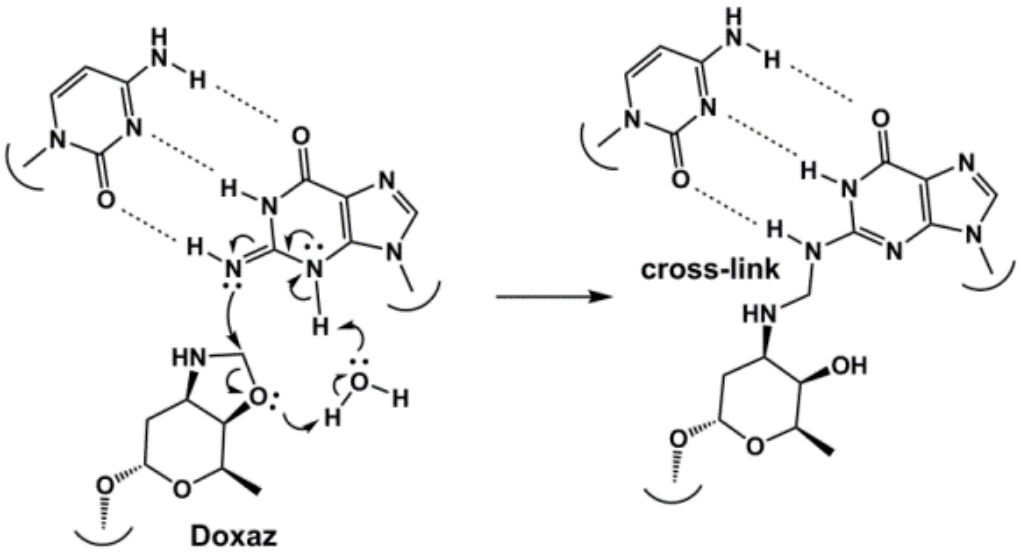


Figure 4: Doxaz mechanism of crosslinking DNA at 5'-GC-3' is executed via the methylene carbon. Figure courtesy of Dr. Ben Barthel.

An ideal candidate for prodrug release is short lived and highly toxic. At 37°C and pH 7.4, Doxaz has a half-life of 3 min, during which the oxazolidine ring hydrolyzes, reforming Dox.[11] Carbamoylation at the oxazolidine ring extends the lifetime and keeps the drug in its inactive form.[16,18] Doxaz exhibits all characteristics for an ideal prodrug candidate, thus substantiating our reasoning for selecting this compound to be the cytotoxic agent.

A prodrug with MARCKS peptide and Doxaz, coupled by a photolabile linker, has never before been synthesized. We attempted to execute the synthesis with a combination of reactions that do not involve harsh conditions and heavy metal catalysts, as they might increase the possibility of degrading Doxaz or prematurely cleaving the linker. We hypothesized that a click reaction would help achieve said synthetic goal.

Click chemistry is a term given to covalent bond-forming reactions that occur rapidly and selectively under relatively mild conditions.[19,20] Bond formation generally occurs from interaction of HOMO and LUMO orbitals of the reactants. For our purposes, we decided to conjugate an azide (HOMO) and alkyne (LUMO) to form a triazole ring.[20] Different structural

applications of this conjugation are depicted in **Figure 5**. Unlike a linear alkyne, the cyclooctyne conjugation does not require a copper catalyst due to the existing ring strain.[19,20] This metal free click reaction was applied to the synthetic scheme of our project.

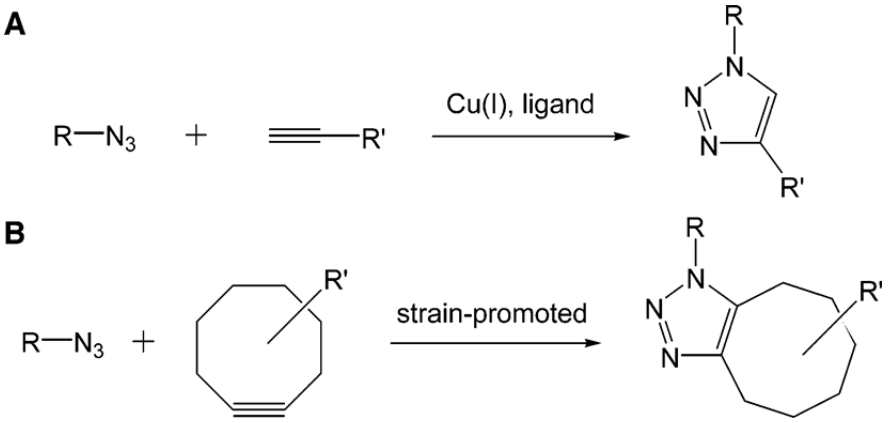


Figure 5: Applications of azide-alkyne click chemistry with a) a terminal alkyne, which requires a Cu(I) catalyst and b) a cyclooctyne, which is dependent solely on angular strain.[19] Figure courtesy of Nicholas J. Agard, Jennifer A. Prescher, and Carolyn R. Bertozzi.

The click reaction commenced with various synthetic attempts. This thesis describes a synthetic route that finally succeeded to yield the desired construct. NMR, HPLC and ESI-MS verify the prodrug's structure. In addition, this thesis also discusses Doxaz-MARCKS as a prominent chemotherapeutic based on in-vitro studies. The anthracycline's anti-tumor properties together with exosome localization abilities of MARCKS as well as the photolabile linker grant this prodrug the high cytotoxicity, localization and selective activation, respectively, which ideal prodrugs strive for. Subsequent sections discuss future experiments that we plan on accomplishing to further improve cancer inhibition selectively.

Materials and Methods

1 - General

1.1 - Abbreviations (listed in alphabetical order)

ACN – acetonitrile, CR – Cerenkov Radiation, DBCO = dibenzocyclooctyne, DCM = Dichloromethane, DIEA = N,N-Diisopropylethylamine, DMF = Dimethylformamide, DMEM+ = Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 1% Glutamax, and .1% Puromycin, DMEM- = non-supplemented, DMSO = Dimethyl Sulfoxide, Dox – Doxorubicin, Doxaz – Doxazolidine, DoxF – Doxoform, ED – effector domain, EDTA - Ethylenediaminetetraacetic acid, EM – exact mass, ESCRT - endosomal sorting complex required for transport, ESI-MS – Electronspray Ionization Mass Spectrometry, EtOH = ethanol, Fmoc = Fluoronylmethyloxycarbonyl chloride, FBS – Fetal Bovine Serum, FDG - 2-deoxy-2-[^{18}F] fluoro-D-glucose, HATU = 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate, HOBt = Hydroxybenzotriazole, HPLC – High Performance Liquid Chromatography, IC_{50} – half maximal inhibitory concentration, Matrix-assisted laser desorption/ionization mass spectrometry, MARCKS – myristoylated alanine-rich C kinase substrate protein, MDA-MB-231 - human breast cancer cell line, MDR – multidrug resistance, MeOH = methanol, mqH2O = Milli-Q-water, NMP = N-Methylpyrrolidone, NMR – Nuclear Magnetic Resonance, NTA - Nanoparticle Tracking Analysis, P170GP - P170 glycoprotein efflux pump, PBS – phosphate buffered saline, PET – positron emission tomography, PG – protecting group, PPD - pentyl PABC-Doxaz prodrug, PS – phosphatidylserine, RT - room temperature, SDS = Sodium dodecyl sulfate, TFA - Trifluoroacetic acid, TIPS = triisopropylsilane

1.2 - Chemicals and Cell Lines

Reagents were ordered from Sigma Aldrich, unless otherwise noted. Doxorubicin hydrochloride was a gift from the Koch Lab. DMEM, FBS, pyromicin and trypsin-EDTA were ordered from Gibco Life Technologies. ExoQuick-TC kit was ordered from System Biosciences. The photolabile linker used was synthesized by graduate student, Ryo Tamura. All peptides were synthesized using the CEM Liberty Microwave Peptide Synthesizer via solid phase Fmoc peptide chemistry. The resin used was H-Rink-Amide-Chem matrix, with a loading capacity of 0.45 mmol/g, ordered from PCas BioMatrix Inc. Amino acids were purchased from ChemPep Inc. ^{1}H-NMR spectra were generated with Bruker AV-III 400 MHz NMR Spectrometer. Reverse phase HPLC was performed with an Agilent Technologies 1200 Series instrument. Analytical HPLC samples were injected onto 250 x 4.6 mm C18 reverse-phase (ODS) column, eluting at 1 mL/min. Purification was performed using the C18 250x10 mm column, eluting at 3 mL/min. UV-vis spectroscopy was performed with a Beckman Coulter DU 730 Life Science UV/Vis Spectrophotometer. ESI-MS analysis was performed using a Thermo Finnigan LCQ LC/MS Mass Analyzer. An OCRA-Flash 2.8 C11440 Hamamatsu Digital Camera was used for exosome analysis. A Bright Line Reichert hemocytometer with 0.1 mm depth was used to count cells. A General Electric monochromatic UV lamp of 350 nm was used for the irradiation procedure. Evos FL Life Technologies fluorescent microscope was used to monitor prodrug endocytosis and generate photography. The 96 well plates were read with a Beckman Coulter DTX 880 Multimode Detector. GraphPad Prism Program was used to calculate IC_{50} values.

The MDA-MD-231 cell line (University of Chicago) was maintained in DMEM medium, supplemented with 10% FBS, 1% Glutamax, and 0.1% Puromycin. Cells were incubated at 37°C in an atmosphere of 95% air and 5% CO_2, unless otherwise noted.

2- Synthesis, Purification and Characterization of Prodrug

2.1 - Dox Extraction

A clinical sample of Dox•HCl (40 mg/ 100 mg lactose) was dissolved in 10 mL MeOH. The pH was adjusted to 8.5 with the $NaHCO_3$/Na2HCO_3 buffer. The resulting solution was extracted with 20 mL of chloroform. The procedure was repeated twice. The organic layers were combined and dried with sodium sulfate. Chloroform was removed via rotary evaporation at 37°C. Dox free base was stored at -19°C.[11] ^{1}H NMR of Dox: (400 MHz, $CHCl_3$) δ 13.75 (1H, s, Ar-O*H*), 12.92 (1H, s, Ar-O*H*), 8.03(1H, d, 1), 7.81 (1H, t, 2), 7.23 (1H, dd, 3), 5.48 (1H, t, 1'), 5.31 (1H, dd, 7), 5.31 (1H, dd, 9-O*H*), 4.75 (2H, s, 14), 4.08 (3H, s, 4-OMe), 4.00 (1H, dq, 5'), 3.25 (1H, t, 14-O*H*), 3.04 (2H, dd, 10), 2.90 (1H, s, 4'-O*H*), 2.36 (2H, d, 3'-NH_2), 2.15 (1H, dt, 3'), 1.75 (2H, td, 8), 1.66 (2H, dt, 2'), 1.34 ppm (3H, d, 5'-Me).

2.2 - Doxaz Synthesis

Dox free base was dissolved in chloroform, followed by addition of paraformaldehyde (100 equiv.). The reaction was stirred at RT, under degased conditions with argon gas, and monitored by ^{1}H-NMR. Completion was observed after 3 days. ^{1}H-NMR indicated that DoxF, a dimer of Doxaz, was the major product. This was an expected outcome due to the excess paraformaldehyde added. DoxF hydrolyzes to Doxaz upon addition of water, so the crude mixture was utilized for proceeding steps.[3,11] ^{1}H NMR of DoxF: (400 MHz, CHCl3) δ 13.75 (1H, s, Ar-O*H*), 12.92 (1H, s, Ar-O*H*), 7.85 (1H, dd, 1), 7.69 (1H, t, 2), 7.39 (1H, dd, 3), 5.52 (1H, dd, 1'), 5.10 (1H, dd, 7), 4.86 (1H, s, 9-O*H*), 4.76 (2H, s, 14), 4.73 (1H, d, ox), 4.21 (1H, d, ox), 4.04 (1H, dq, 5'), 3.98 (1H, dd, 4'), 3.90 (3H, s, 4-OMe), 3.52 (2H, s, NCH_2N), 3.40 (1H, dt, 3'), 3.04 (1H, bs, 14-O*H*), 3.04 (2H, dd, 10, J = 2, 19 Hz), 2.86 (2H, d, 10, J = 19 Hz), 2.50 (2H,

dt, 8, J = 3, 15 Hz), 2.19 (2H, dt, 2'), 1.92 (2H, dd, 8, J = 15, 2 Hz), 1.77 (2H, ddd, 2'), 1.36 ppm (3H, q, 5'Me).

2.3 - Synthesis of Doxaz-N3

Doxaz (0.075 mmol, 1 equiv.) and 2 equiv. of linker and HOBt (0.150 mmol) were used to synthesize Doxaz-N_3. The photolabile linker was synthesized by graduate student, Ryo Tamura, with determined 96% purity. Each reactant was dissolved in 1 mL NMP. Molecular sieves were added to the linker solution, followed by step-wise addition of HOBt. The resulting mixture was stirred for 15 min at RT, after which Doxaz was added. The reaction was stirred overnight at RT and its progress was monitored with NMR. ^{1}H NMR of Doxaz-N_3: (400 MHz, CHCl3) δ 13.96 (1H, s, Ar-O*H*), 13.24 (1H, s, Ar-O*H*), 8.08 (1H, d, 1), 7.79 (1H, s, 2), 7.61 (1H, s, Ar), 7.44 (1H, d, 3), 7.03 (1H, t, N*H*), 7.00 (1H, s, Ar), 6.48 (1H, q, Bn), 5.49 (1H, t, 1'), 5.32 (1H, t, 7), 5.05 (2H, s, ox), 4.75 (2H, d, 14), 4.67 (1H, s, 9-O*H*), 4.52 (2H, s, -CH2-), 4.16 (1H, q, 5'), 4.12 (1H, q, 4'), 4.10 (3H, s, 4-OMe), 4.04 (1H, dt, 3'), 3.91(3H, t, PEG), 3.67 (9H, t, PEG), 3.66 (3H, s, Ar-OMe), 3.55 (2H, t, PEG), 3.34(2H, t, PEG), 3.27 (1H, dt, 10), 3.07 (1H, dd, 10), 2.96 (1H, t, 14-O*H*), 2.48 (1H, dt, 8), 2.20 (1H, dd, 8), 1.69 (3H, d, Bn-CH_3), 1.38 ppm (3H, d, 5'-Me). Product analysis was performed with HPLC: flow rate, 1 mL/min; eluent A = Potassium Phosphate Monobasic Buffer, pH 4.6 and eluent B = HPLC grade ACN; gradient, 80:20 A/B at 0 min to 25:75 A/B at 55 min, isocratic to 65 min, back to 80:20 A/B at 70 min, isocratic to 80 min. Eluent was monitored at 210 nm, 254 nm, 280 nm and 480 nm.

2.4 - Quantum Yield of Photolabile Linker

Concentration of Doxaz-N_3 was determined via absorbance (λ = 480 nm, ε = 11500 $M^{-1}cm^{-1}$). A sample (0.39 mol) was dissolved in 1 mL of ACN and transferred to a cuvette. A 12.4 mW Omnichrome HeCd was used to irradiate the solution for 30 min at 325 nm. Aliquots of 10

uL were collected at different times and analyzed by HPLC to determine the % Dox in relation to that of Doxaz-N_3. The following factors were used to calculate quantum yield: power (without cuvette) = 12.4 mW, (with cuvette) = 10.7 mW, incident light = 11.55 mW; energy = 0.462 J, $7.55x10^{17}$ photons; t = 40 sec when % Dox = 9.2. A triplicate experiment was performed.

2.5 - Synthesis of Doxaz-Maleimide

0.0117 mmol (500 mg) of DBCO-Maleimide was dissolved in 500 uL of NMP. 0.03 mM of crude Doxaz-N_3 (in 2.5 mL NMP) was added in a drop-wise fashion. The reaction was foiled and left overnight at RT. The product was dried, lyophilized and stored at -19°C. Purification was performed with HPLC: flow rate, 1 mL/min; eluent A = potassium phosphate monobasic buffer, pH 4.6 and eluent B = HPLC grade ACN; gradient, 80:20 A/B at 0 min to 25:75 A/B at 45 min, isocratic to 50 min, back to 80:20 A/B at 55 min, isocratic to 60 min. Eluent was monitored at 210 nm, 254 nm, 280 nm and 480 nm. The molar amount of product was quantified via absorbance (λ = 480 nm, ε = 11,500 $M^{-1}cm^{-1}$).

2.6 - Peptide Synthesis and Thiol Coupling

1.0 mg of peptides (MARCKS and C2BL3L) were synthesized via Fmoc chemistry with the peptide cyclizer. MARCKS sequence: KKKKKRFSFKKSFKLSGFSFKKNKK; C2BL3L sequence: GGDYDKIGKNDA. 0.075 mmol of MARCKS, 0.150 mmol of S-trityl-3-mercaptopropionic acid and 0.150 mmol HATU were mixed and dissolved in 5.0 mL of DMF. After the solution reached a homogeneous appearance, 0.15 mmol DIEA was added. The reaction was left to stir overnight at RT. Reaction contents were washed with DCM and MeOH, 5 times each. A small amount of product was used for a Kaiser Test to ensure reaction efficiency. 1 drop of each was added: ninhydrin, phenol in EtOH, pyrimidine, and the resulting mixture was heated at 90-100°C for 5 min. A yellow solution indicated success of the reaction; a

purple solution indicated failure of thiol conjugation. The peptide was cleaved from the resin with TFA solution: 50 uL TIPS, 125 uL mqH2O, 125 uL EDT, and 4.7 mL TFA.[6] The reaction was left to stir at RT for 2 hr. Ether was used to precipitate the peptide. It was then lyophilized and stored at -19°C. Reverse phase HPLC was used to purify each peptide. The frozen solid was dissolved in 1 mL H_2O (0.1% TFA), and the following method was applied: flow rate, 3.0 mL/min; eluent C = 95% H_2O, 5% ACN, 0.1% TFA, pH 2.0 and eluent D = 100% ACN, 0.1% TFA; gradient, 95:5 C/D at 0 min to 50:50 C/D at 45 min, isocratic to 50 min, back to back to 95:5 C/D at 55 min, isocratic to 60 min. Eluent was monitored at 210 nm, 254 nm and 280 nm. The identity of the purified peptides was analyzed by ESI-MS.

2.7 - Doxaz-MARCKS Synthesis

1.0 umol MARCKS was dissolved in 700 uL of N_2-degased PBS buffer. 1.0 umol of Doxaz-Maleimide solid was dissolved in 400 uL DMF and added to the PBS mixture. The reaction was foiled and left overnight at RT, while maintained in a N_2 atmosphere. The product was dried, lyophilized and stored at -19°C. Purification was performed with reverse phase HPLC. The solid was dissolved in 0.5 mL DMF and 1.0 mL H_2O (0.1% TFA). The following method was used: flow rate, 3 mL/min; eluent C = 95% H_2O, 5% ACN, 0.1% TFA, pH 2.0 and eluent D = 100% ACN, 0.1% TFA; gradient, 95:5 C/D at 0 min to 80:20 C/D at 30 min, isocratic to 60 min, to 50:50 C/D at 65 min, isocratic to 75 min, back to 95:5 C/D at 80 min, isocratic to 90 min. Eluent was monitored at 210 nm, 254 nm, 280 nm and 480 nm. The molar amount was quantified via absorbance (λ = 480 nm, ε = 11,500 $M^{-1}cm^{-1}$). ESI-MS was used to verify the identity of the purified Doxaz-MARCKS.

3 - Cell Experiments

3.1 - Exosome Size Analysis

Cells were grown in supplemented DMEM (DMEM+) until 80% confluence. Media was changed to non-supplemented DMEM (DMEM-) in order to generate cell-derived exosomes, not those of FBS. Cells were incubated for 2 days in these conditions, then centrifuged at 3000 x g for 15 min. Pellet was discarded and 2 mL of ExoQuick-TC was added to the isolated supernatant. The solution was kept refrigerated overnight, then centrifuged at 1500 x g for 1hr. The resulting pellet was dissolved in 500 uL PBS. Exosomes were analyzed via Nanoparticle Tracking Analysis (NTA), using the Stokes-Einstein equation, to determine diameter size and concentration released by MDA-MD-231 cells.[8]

3.2 - IC_{50} Assays

96 well plates were used to generate IC_{50} values of Doxaz-MARCKS and Doxaz-C2BL3L. Cells were cultured in DMEM+, counted with hemocytometer, and distributed to inside wells of plate at ~1000 cells/well (100 uL). 100 uL of PBS was transferred to outside wells. Plates were incubated for 24 hr to allow adherence. The prodrugs (Doxaz-MARCKS and Doxaz-C2BL3L control) were dissolved in PBS to generate the following final concentrations: 1 nM, 10 nM, 100 nM, 1 uM, 4 uM, 7 uM and 10 uM. 25 uL of the corresponding prodrug solution was added at appropriate times to achieve designated incubation periods (6, 12, and 24 hr). Two lanes were designated for control: 25 uL PBS and 50 uL DMSO. Wells were washed with PBS and 50 uL of fresh PBS was added prior to irradiation. The plate designated for UV irradiation was placed 12 cm under the UV lamp (λ_{max} = 350 nm, P = 6 mW) without its lid; the non-UV plate was foiled. Plates were irradiated or foiled for 30 min after which they were kept at RT for an additional 30 min. Various wells were imaged by a fluorescence microscope, followed by two 100 uL PBS washes and finally addition of 125 uL fresh DMEM+. Plates were incubated until control wells reached ~80% confluence (3-5 days). Crystal violet was utilized to

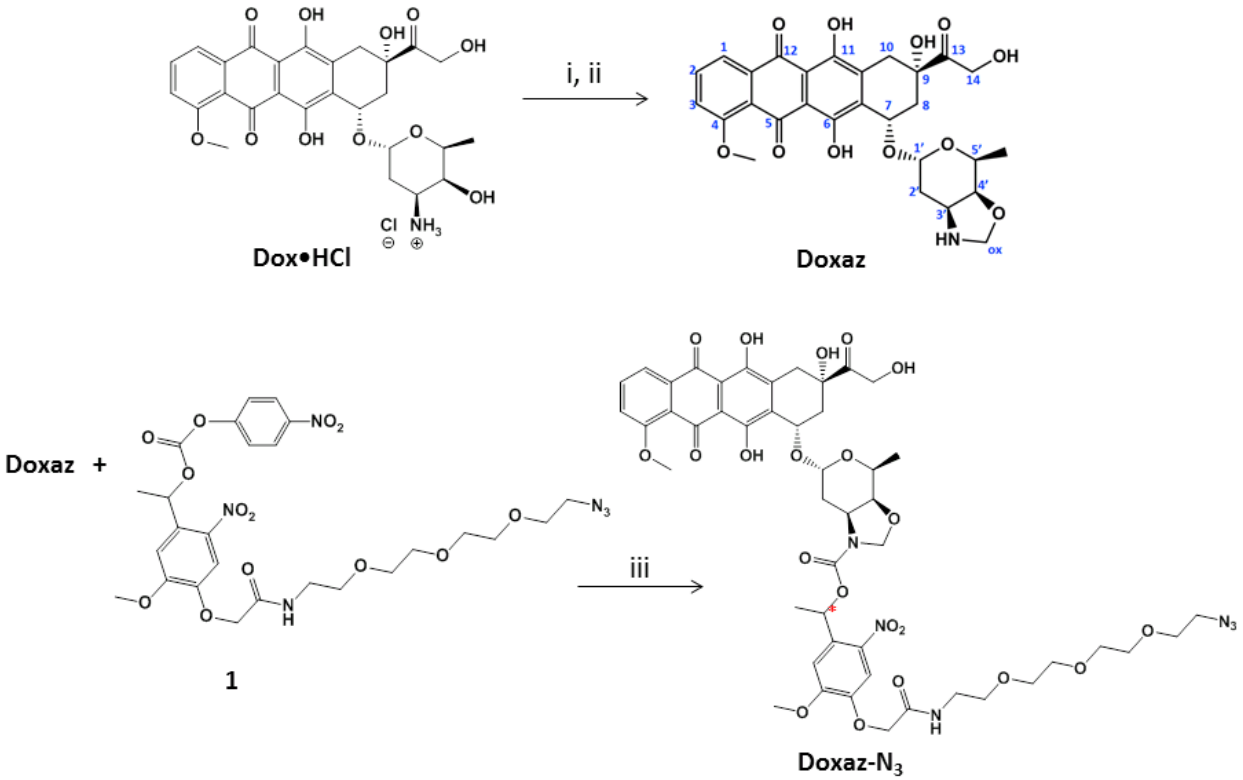
quantify cells. 5% formalin in PBS (5-10 min) was used to fix cells, followed by addition of 50 uL of 0.1% crystal violet in H_2O (20 min) for staining, then 100 uL of 1:1 isopropyl alcohol : 2% SDS in dH_2O (30 min) for solubilizing. Optical density was measured at 570 nm by the plate reader. IC_{50} values were generated from GraphPad Prism. The experiment was performed in triplicate. Each drug concentration was executed twice per incubation period.[11]

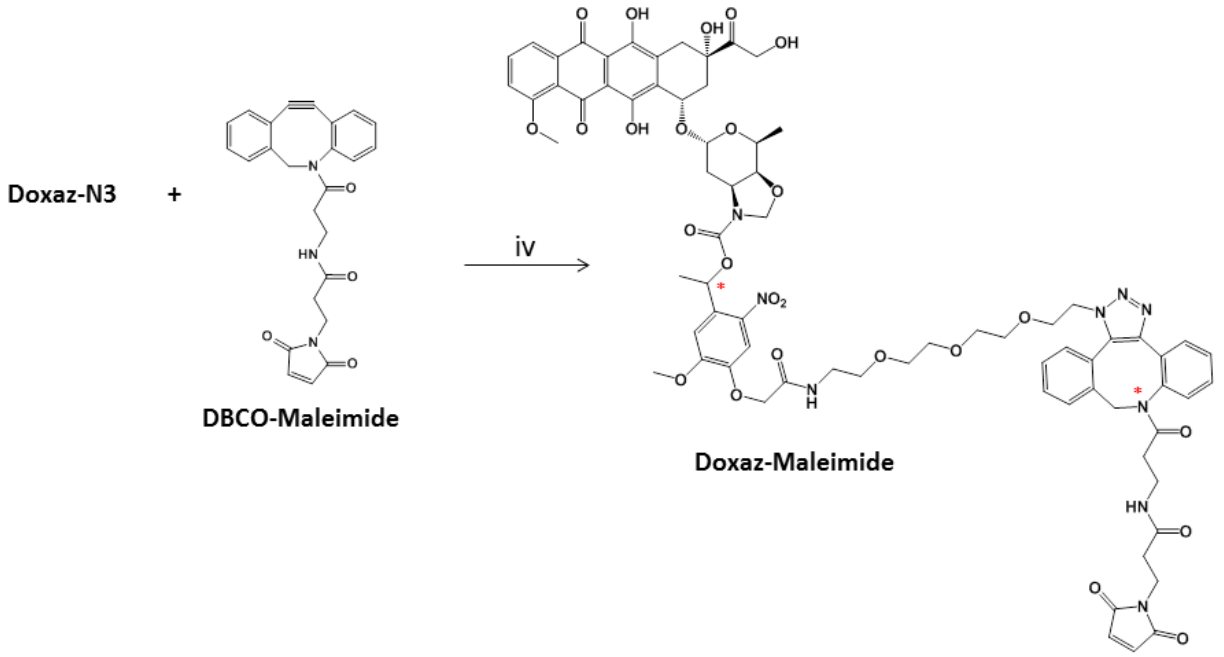
Results

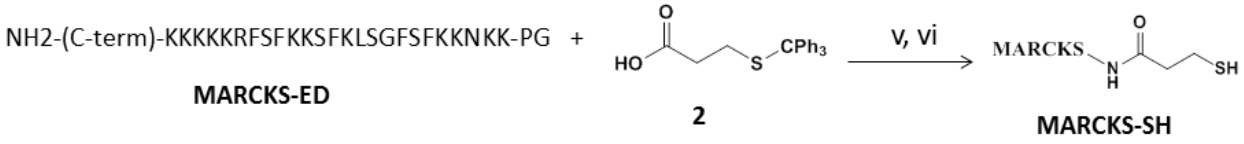
1 - Prodrug Synthesis

In this experiment, we hypothesized that synthesis of a prodrug with the components Doxaz and MARCKS interlinked by a photolabile group would be possible. The following section describes the results of the synthesis, which confirm the original hypothesis. **Figure 6** demonstrates the synthetic scheme, which annotates the steps required to achieve the final construct.

Figure 6: Synthetic scheme of Doxaz-MARCKS prodrug. (i) $NaHCO_3/Na_2CO_3$, RT; (ii) paraformaldehyde, RT; (iii) HoBT, NMP, RT; (iv) NMP, RT; (v) HATU, DIEA, DMF, RT; (vi) TFA, TIPS, EDT, mqH_2O, RT; (vii) PBS, N_2, RT. The carbon labeling shown on Doxaz will be referenced. Centers that give rise to regio-isomers and diastereomers are labeled with red stars.



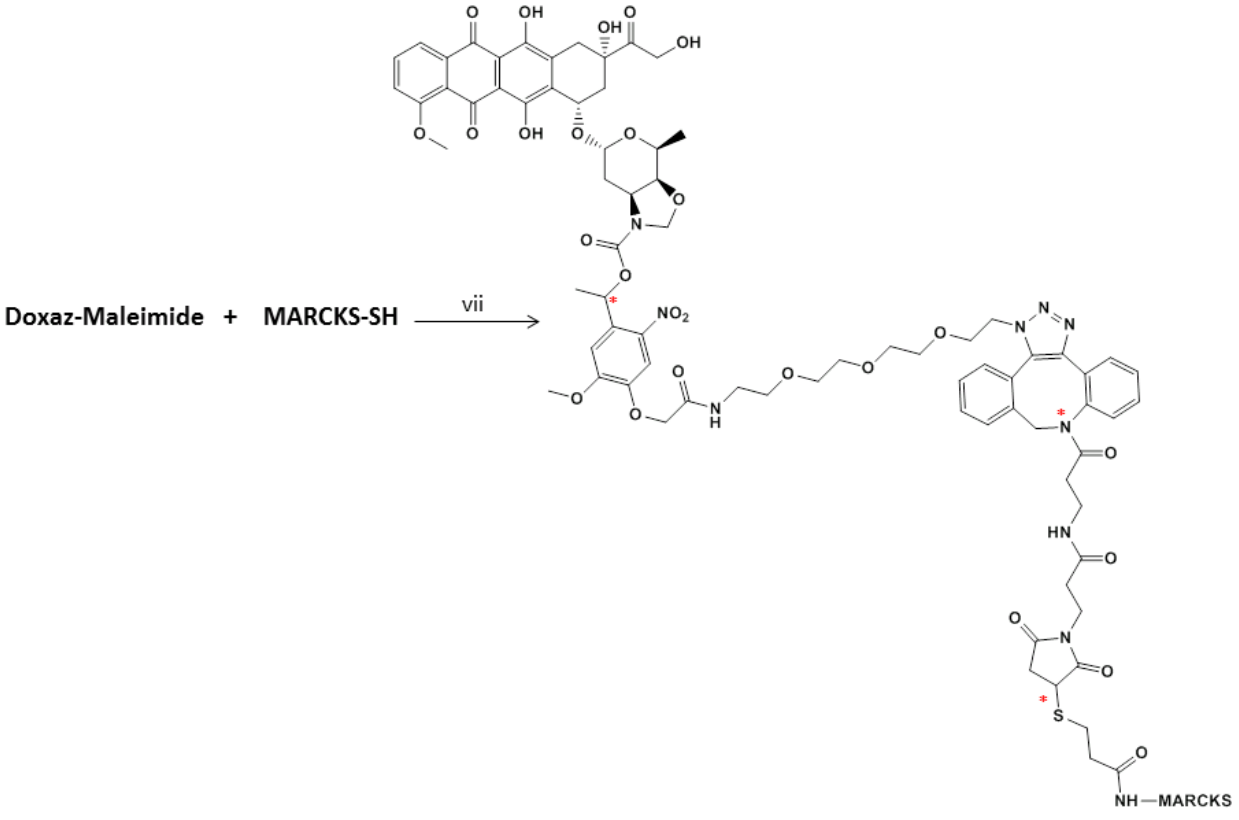




MARCKS-ED

2

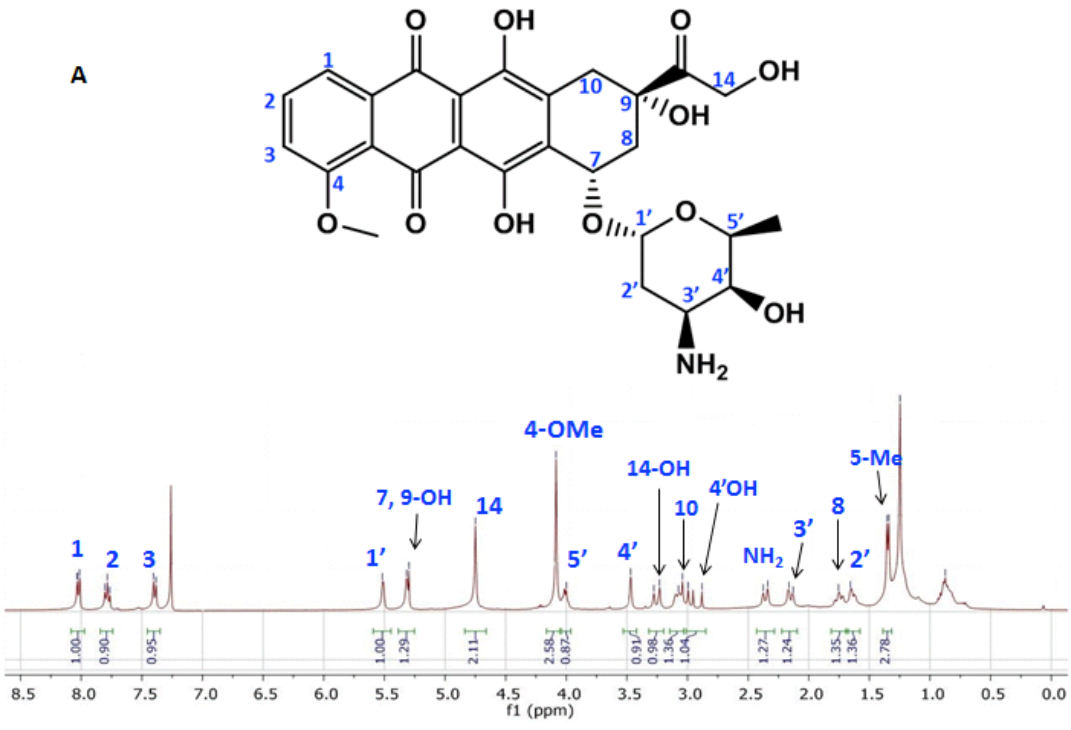
MARCKS-SH

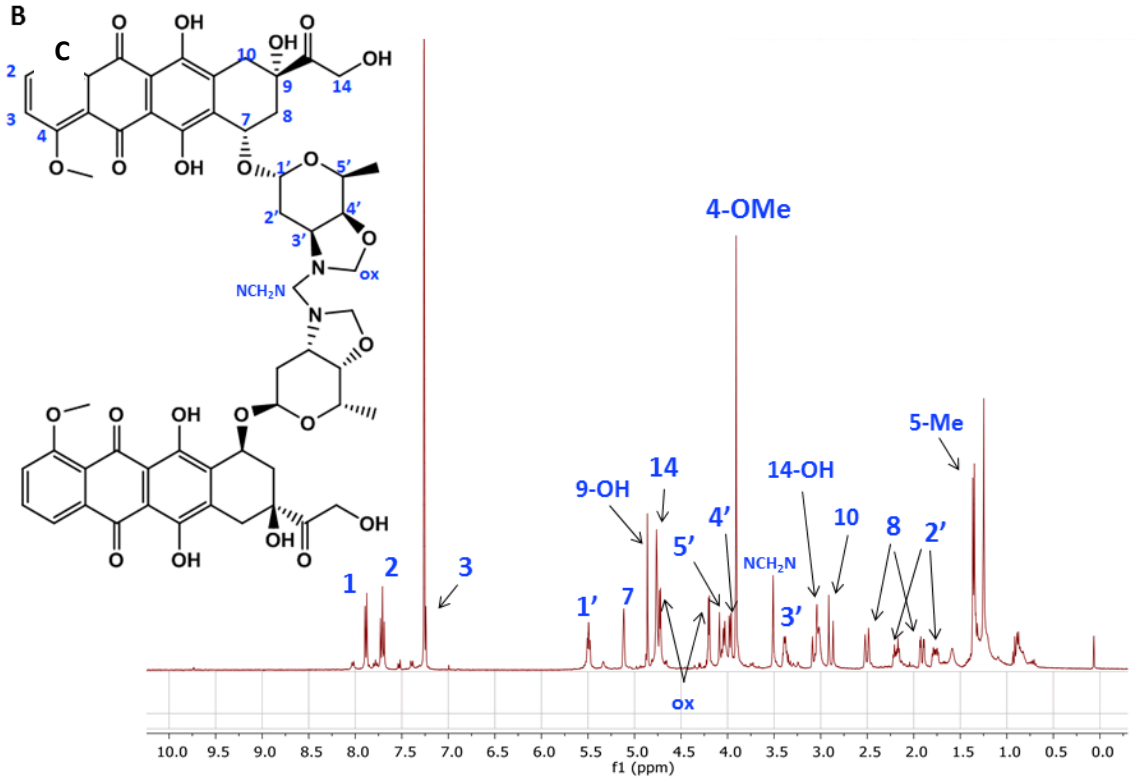


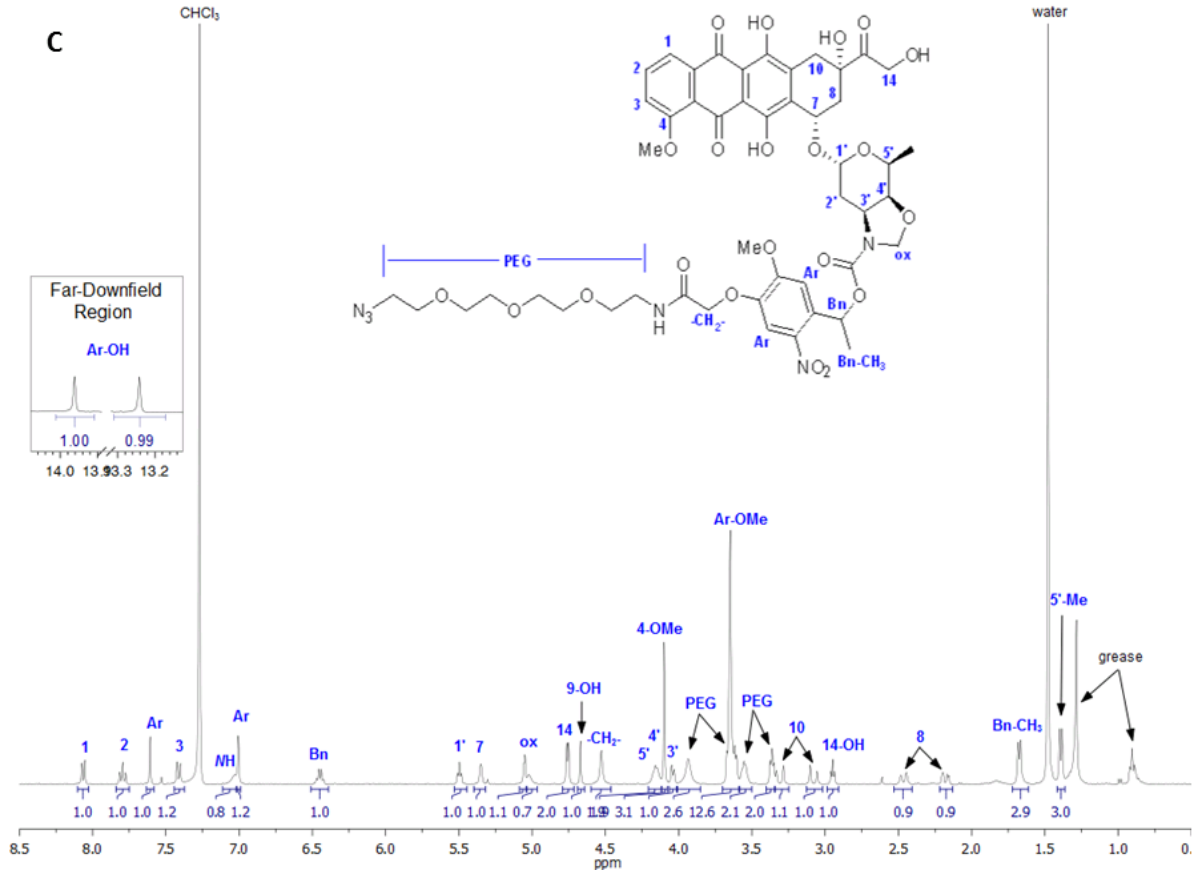
1.1 - Doxaz Preparation

The scheme commenced by neutralizing the salt form of Dox to extract the Dox free base into chloroform. Its ^{1}H-NMR spectrum is shown in **Figure 7a**. Chemical shifts assignments for all NMR spectra are detailed in the methods section; the hydrogen numbering schemes are as shown in the figure. Upon addition of excessive paraformaldehyde, Dox free base is converted to DoxF, a dimer of Doxaz. We expected this dimerization due to previously reported results by the Koch group.[3,11] The formation of DoxF was monitored by ^{1}H-NMR (**Figure 7b**); the appearance of the 'ox' peaks at 4.21 and 4.73 ppm determined the success of the reaction. Upon addition of small amount of water, DoxF was hydrolyzed to Doxaz.[3,11] Thus dimer formation was not quantified and the crude material was utilized for the next synthetic step, assuming that the dimer would hydrolyze to its monomeric state.

Figure 7: 1-D proton NMR spectra of a) Dox, b) DoxF, and c) Doxaz-N_3. Phenolic hydrogens (Ar-OH) are not shown in a) and b). Data generated by Ryo Tamura and figure courtesy of Ryo Tamura and Price Kirby.




B
C
4-OMe
5-Me
14
9-OH
14-OH
10
8
2'
5'
4'
NCH2N
1
2
3
1'
7
3'
ox
f1 (ppm)


C
CHCl3
water
Far-Downfield Region
Ar-OH
1.00
0.99
PEG
Ar-OMe
4-OMe
5'-Me
grease
9-OH
14-OH
Bn-CH3
ppm

1.2 - Synthesis of Doxaz-N_3

Carbamoylation was used to generate Doxaz-N_3, a carbamate derivative that is stable in aqueous conditions. Due to the stable leaving group, para-nitrophenol, of the photolabile linker (1), its attachment to Doxaz was a facile reaction that did not create significant potential for undesired side reactions nor did it require addition of harsh reagents. The linker contained a 3-PEG spacer, which was inserted to increase the construct's hydrophilicity and couple the caged linker to the terminal azide.

Identity of Doxaz-N_3 was ensured via ^{1}H-NMR (**Figure 7c**). Phenolic peaks at 13.96 and 13.24 ppm indicate that the Doxaz moiety was not degraded during the reaction and that the phenolic hydrogens did not undergo an undesired side reaction. Also, presence of the 'ox' peak (5.05 ppm) verifies that Doxaz did not hydrolyze back to Dox.

Manifestation of Doxaz-N_3 was confirmed by analytical HPLC (**Figure 8a**), which revealed two peaks for the azides, with retention time of 39 min. These are the two diasteriomers (marked with a red *), which are resulting from the benzylic stereocenter. Removing this methyl would not interrupt the photodissociation. However, the presence of the methyl gives rise to a tertiary carbon radical in the photochemical mechanism (**Figure 2**), hence stabilizing the intermediate more than that of an otherwise secondary carbon radical. We assumed that the stereochemistry should be irrelevant to the photodissociation mechanism.

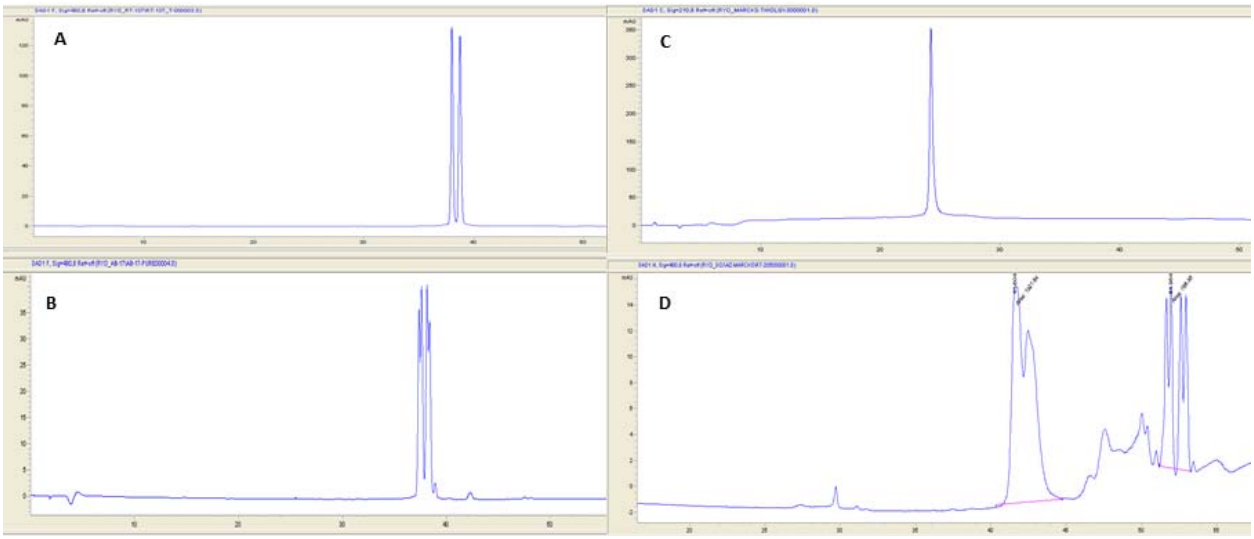


Figure 8: HPLC spectra of a) Doxaz- N_3, b) Doxaz-Maleimide, c) MARCKS-SH and d) Doxaz-MARCKS. Number of peaks corresponds to the number of diastereomers and regio-isomers. A, B and D were monitored at 480nm, C at 210nm.

1.3 - Quantum Yield of Linker

Doxaz-N_3 was irradiated with a 12.4 mW monochromatic laser at 325nm for 30 min. Aliquots were collected throughout to determine the amount of Doxaz-N_3 in relation to released Dox (**Table 1**), which was quantified by HPLC. Ryo Tamura collected this data. Columns designated as A and B refer to the diastereomer peaks shown in the HPLC of Doxaz-N_3 (**Figure 8a**). At the 40 sec time increment, 9.2% of Dox was released; this data point was used to calculate the quantum yield. A triplicate average resulted in a quantum yield of 0.025. This value is in the low end of the quantum yield range, yet this is beneficial for our purposes because it signifies that UV must be applied for longer durations prior to release of the cytotoxic agent. This makes administration safer and synthesis more practical.

Time (sec)	Dox	Doxaz-N3 (A)	Doxaz-N3 (B)	% Dox
0	0	482.9	533.8	0
20	52.6	483	535.6	4.91
40	102	475	526.5	9.243
60	146.6	480.6	531.9	12.648
80	190.9	457.3	512.4	16.448
900	1071.1	102	117.8	82.973
1800	1309.3	Combined 48.7		96.414

Table 1: Photolysis experiment of Doxaz-N_3. Amount of each compound present at different times was determined by HPLC analysis. A and B refer to HPLC diastereomer peaks of Doxaz-N_3. The t=40 sec data point was used to calculate quantum yield of the photolinker (highlighted in blue).

1.4 - Doxaz-Maleimide

The crude fraction of Doxaz-N_3 was used to form Doxaz-Maleimide; 30% yield was achieved after HPLC purification. Four peaks appeared as expected (**Figure 8b**), representing the four predicted isomers. The retention time is 38 min. A phosphate buffer, rather than acidic TFA, was utilized for this purification in order to prevent hydration of the maleimide moiety.

A copper free click reaction was used to conjugate Doxaz-N_3 and the commercially available DBCO-Maleimide. The intrinsic angular strain of the cyclooctyne ring, in addition to electron withdrawing groups, such as the benzo rings, allow faster distortion of the transition state, hence considerably increasing the reaction rate.[19,20] It is speculated that this effect lowers the LUMO energy level of the alkyne in relation to that of the azide's HOMO.[21]

As mentioned earlier, a less hydrophobic group, such as a terminal alkyne, could have served as an alternative for this conjugation. In this case, a Cu(I) catalyst must be applied analogously with sodium ascorbate, a reducing agent, to prevent oxidation to Cu(II). This step was attempted yet proved unsuccessful. It was speculated that the reducing agent degraded Doxaz by reducing its carbonyls at C5 and C12 to alcohols (**Figure 6**). Furthermore, various

studies stated that copper is toxic to cells, thus an additional dialysis step would be required prior to cell administration to remove this metal. The cyclooctyne reaction, in which a metal catalyst is not required, did not degrade the anthracycline. A clean HPLC spectrum, monitored at 480 nm, verified that our prediction was confirmed (**Figure 8b**).

1.5 - Peptide preparation

The MARCKS peptide and C2BL3L (sequence: GGDYDKIGKNDA) were synthesized with a peptide cycler via Fmoc chemistry. Both were loaded onto an H-Rink-Amide-Chem resin. The subsequent thiol conjugation was a two-step reaction: the conjugation itself and deprotection. Yield summed to 5% after HPLC purification; retention time = 24 min (**Figure 8c**). ESI-MS verified the identity of the peptide. The peak of highest intensity, 792.73 m/z, corresponded to the M+4 fragmentation of MARCKS-SH and its exact mass (EM) of 3165.88 Da (**Figure 9a**).

We relied on the colorimetric Kaiser Test to determine if the thiol coupling was successful. It was applied between steps (v) and (vi). This analysis is dependent on ninhydrin, which reacts with primary amines to form a purple complex. In absence of primary amines, the solution remains yellow, as was the case after reaction (v) was complete. These results along with the narrow HPLC peak indicate that thiol conjugation occurred almost quantitatively. Thus the low yield is likely a consequence of the HPLC purification or the peptide synthesis itself, which may be hindered due coupling of seven successive cationic residues.

In the future, we may utilize an alternative purification method. For example, ion exchange chromatography would be appropriate due MARCKS' highly charged nature.

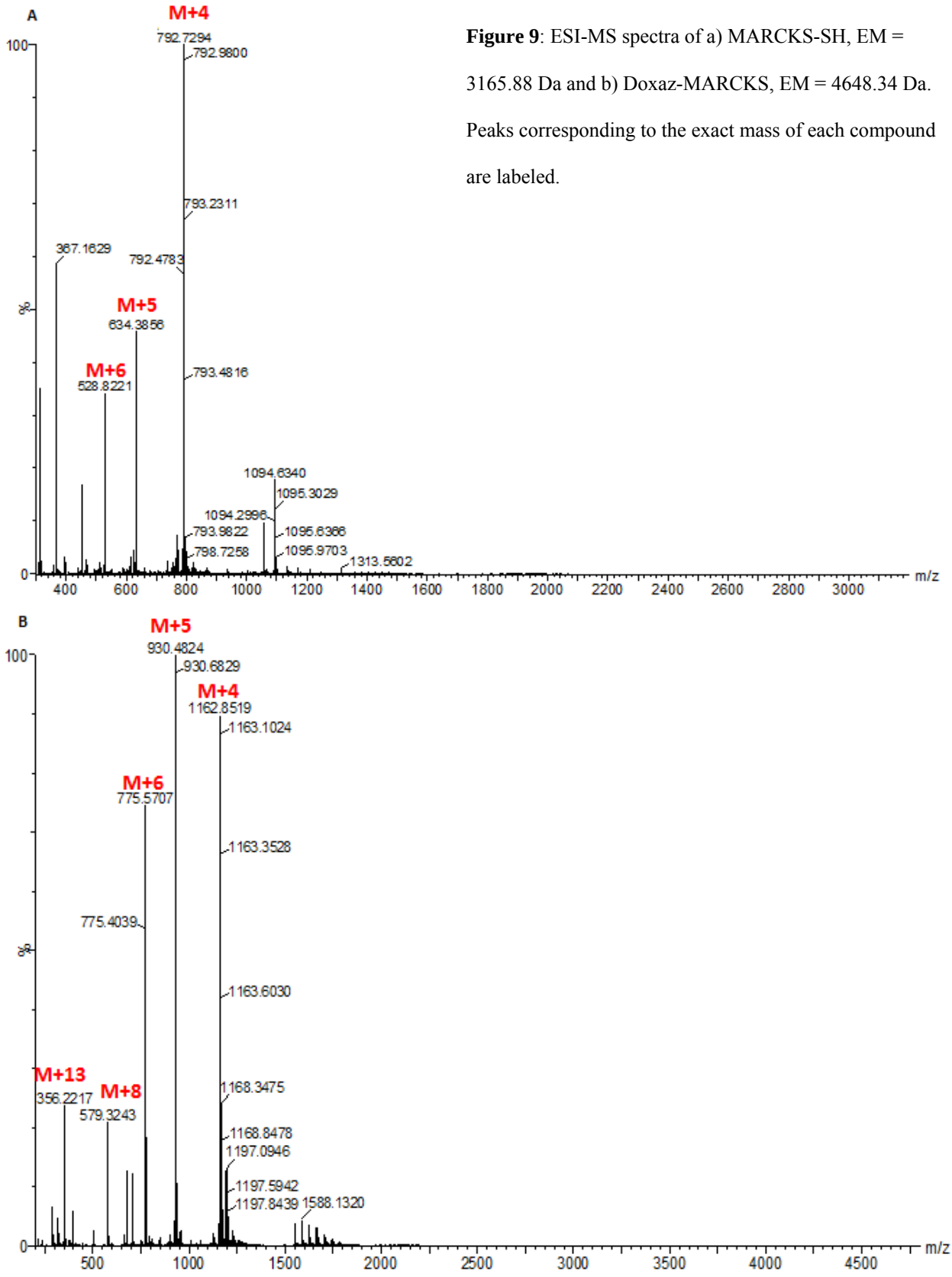


Figure 9: ESI-MS spectra of a) MARCKS-SH, EM = 3165.88 Da and b) Doxaz-MARCKS, EM = 4648.34 Da. Peaks corresponding to the exact mass of each compound are labeled.

1.6 - Doxaz-MARCKS

The Michael reaction of a thiol acting as a nucleophile and attacking the electrophilic double bond of the maleimide, was used to conjugate Doxaz-Maleimide and MARCKS-SH to form Doxaz-MARCKS. The reaction was carried out under degased conditions to prevent disulfide bond formation since oxygen has mild oxidative properties. HPLC was used to purify the product, ensuing in a 66% yield. The retention time was noted at 41-44 min, while the unreacted starting material is present at 51-53 min (**Figure 8d**).

This final construct contained 8 regio and diastereomers for which we expected to see 8 individual peaks; however, the method used produced two broad peaks instead. We speculated that this outcome was due to the HPLC method used, not due to deterioration of the product. ESI-MS confirmed the identity of Doxaz-MARCKS (EM = 4648.34 Da). The peaks of highest intensity, 1162.85, 930.48 and 775.57 m/z, corresponded to M+4, M+5 and M+6 charged states, respectively (**Figure 9b**). Presence of a dark red pigment indicated that the Dox moiety was not degraded.

2 - Cell experiments

Herein, we evaluated the efficacy of the Doxaz-MARCKS prodrug by administering it to MDA-MB-231 human breast cancer and applying UV light. We predicted that there would be a significant difference in IC_{50} values between cells treated with the prodrug and light versus those kept in the dark, as well as cells treated with Doxaz-MARCKS versus those treated with Doxaz-C2BL3L, the control construct.

2.1 - Exosome Analysis

Because the prodrug localization relies on exosomes, those of the MDA-MB-231 cancer cell line were examined. To derive exosomes solely from the cells and avoid collecting those of

FBS, we cultured cells in non-supplemented DMEM (DMEM-) for 2 days. Nanoparticle Tracking Analysis (NTA) determined the average diameter to be 145.9 ± 2.1 nm, the mode 123.6 ± 8.8 nm, and approximately 1740 exosomes released per cell within a 2 day incubation period (**Figure 10**). This analysis is dependent on the Stokes-Einstein equation (also shown in Figure 10) where D is the diffusion coefficient, k_B is Boltzmann's constant, T is temperature, η is fluid viscosity and r is the radius of the particle.

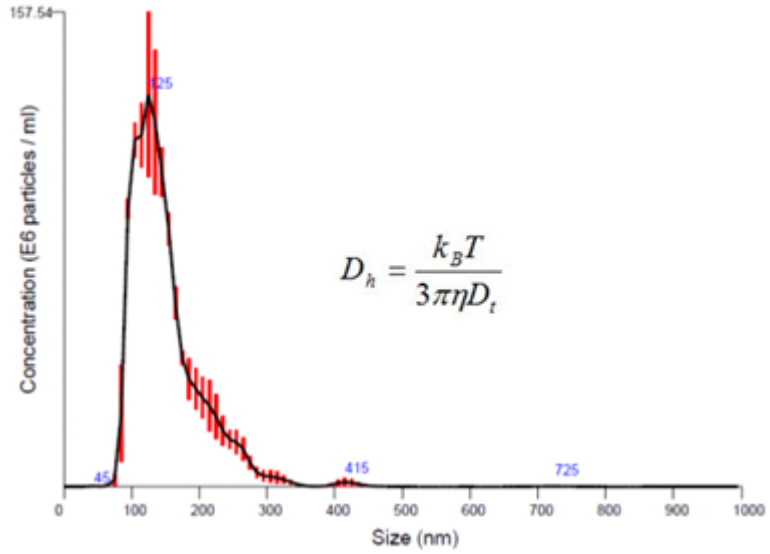


Figure 10: a) Nanoparticle Tracking Analysis (NTA) determined that mostly exosomes of 123.6 ± 8.8 nm diameter are produced by MDA-MB-231 cells. Stokes-Einstein equation was used for this analysis. Data generated by Ryo Tamura.

The nanometric diameter signifies that the MARCKS peptide is capable of binding to the microvesicles of this cell line via curvature sensing. Thus, the mechanism by which the prodrug is predicted to act is feasible. The amount of exosomes generated is a relative number, which will be useful for comparison in future experiments with different cell lines.

2.2 - IC_{50} Assays

The prodrug's efficacy was evaluated by assessing IC_{50} values. Cells were treated for 6, 12, and 24 hr, then incubated for 5 days to allow control wells to reach 80% confluence,

followed by crystal violet staining to determine cell viability. The results are summarized in **Table 2.** The apparatus for irradiation is shown in **Figure 11**. A 6 mW UV lamp was placed 12 cm above the 96 well plate. The negative UV control plate was foiled and placed adjacently.

Incubation Time	Light control	6 hr (nM)	12 hr (nM)	24 hr (nM)
Doxaz-MARCKS	+	11.7	5.7	7.9
	-	4600	1400	1300
Doxaz-C2BL3L	+	970	800	470
	-	4640	4310	2240

Table 2: Summary of IC_{50} values generated by 2 drugs: Doxaz-MARCKS and Doxaz-C2BL3L, each tested with (+) and without (-) UV light for 6, 12, and 24 hr incubation periods.

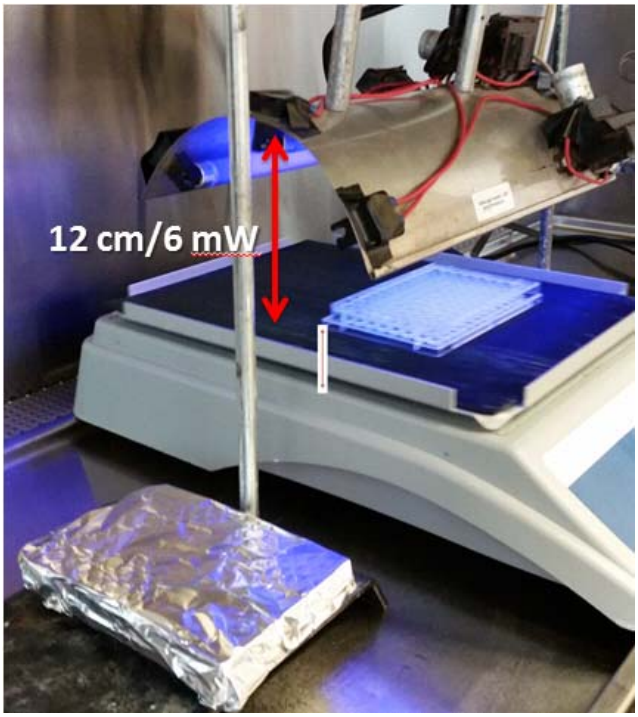


Figure 11: UV lamp set up used for the irradiation protocol. The red arrow indicates a 12 cm distance between the plate and the 6 mW UV source. The (-) UV plate is foiled while the (+) UV plate was irradiated.

As shown, addition of light decreases IC_{50} values in both the C2BL3L control as well as the MARCKS construct, demonstrating the selectivity appropriated by the photolabile linker. Besides the 24 hr incubation data point of Doxaz-MARCKS (+UV), cell viability is proportional to incubation time, again for both the control and the construct of interest. The micromolar IC_{50}

values of the non-UV trials suggest that the photolabile linker may be prematurely cleaved in the endosomal-lysosomal solution. Overall, IC_{50} values for UV-exposed Doxaz-MARCKS were all in nanomolar range, suggesting a potent cytotoxic agent. We anticipated that the same outcome was not observed in C2BL3L construct due to its net negative charge, whereas the features of MARCKS allowed better exosomal localization thus improved uptake.

An analogous experiment was repeated with 1 and 3 hr incubation times. However, IC_{50} values could not be generated due to an inconsistent relationship between cell viability and prodrug concentration. Previous kinetic studies of exosomes estimate that exosome internalization occurs within 2 hr at 37°C.[7] This suggests a minimum prodrug incubation period of 3 hr or longer.

Preliminary pictures demonstrate that Doxaz-MARCKS uptake is dose dependent since the cells treated with 10 uM demonstrated a higher red fluorescence (at 580nm) than those treated with 1 uM (**Figure 12**). These events were captured after prodrug media was replaced with fresh PBS, signifying that the fluorescence observed is due to the endocytosed prodrug.

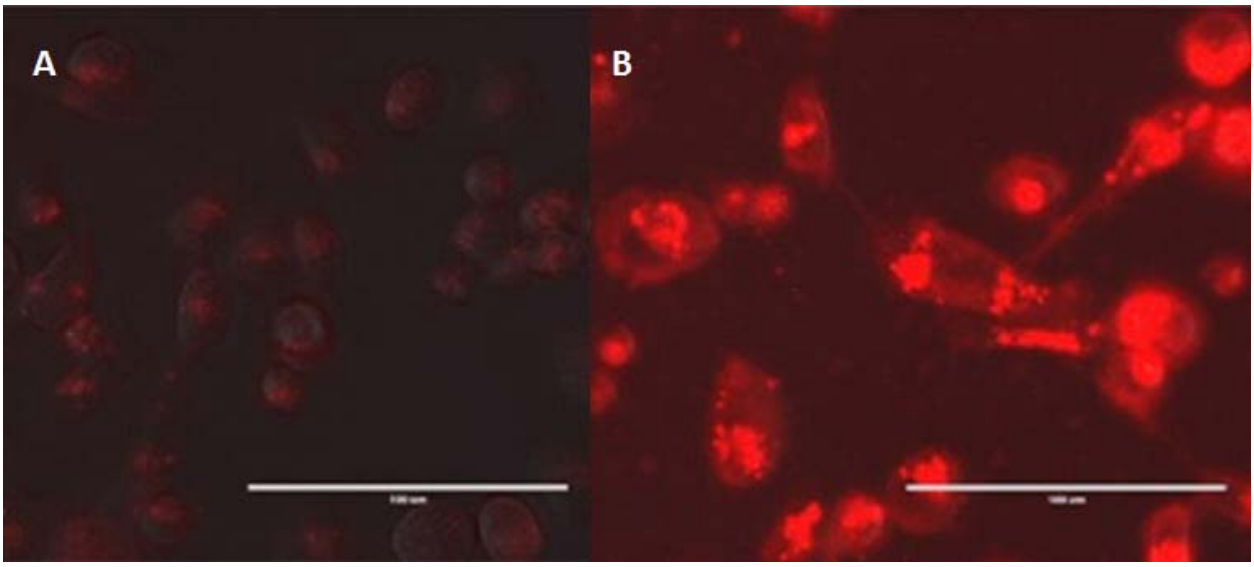


Figure 12: Endocytosis by MDA-MB-231 cells treated with a) 1 uM and b) 10 uM of Doxaz-MARCKS prodrug. Emission was measured at 580 nm.

3 - Discussion

We proposed that the following mechanism occurred upon prodrug administration. The MARCKS portion of the prodrug is localized to exosomes of the MDA-MB-231 cells. The exosomes were then endocytosed into the cell, engulfing the entire prodrug. The media was changed, thus prodrug remaining in the external environment was removed. Later UV application activated the prodrug in the endosome or lysosome, releasing the hydrophobic Doxaz, which, due to its hydrophobic properties, diffused out of the endosomal (or lysosomal) lipid bilayer and through the nuclear envelope, entering the nucleus. There it crosslinked and intercalated DNA at 5'-GC-3' sites, inducing apoptosis.

It is difficult to differentiate the nucleus and the lysosome solely via observation with fluorescence microscopy. In future experiments we plan to dye the nucleus with DAPI and the lysosome with LysoTracker to discern the organelle in which the prodrug and drug are located in at different stages of the experiment. Prior to activation, the prodrug is expected in the lysosome; after UV application, the Doxaz drug is expected in the nucleus.

Furthermore, our future plans include generating IC_{50} values of the Doxaz-MARCKS prodrug in different cell lines, especially in those with the MDR phenotype and P170GP overexpression. Cells vary in their properties, including exosome size and concentration, and sensitivity to UV light. These experiments will determine whether the prodrug is as potent as shown thus far.

4 - Future experiments

Although UV light is a new and innovative selective tool for chemotherapy, it is also a major impediment since it does not penetrate the skin. Therefore, application of this prodrug is limited to external melanoma or that with fiber endoscopy. Since most melanomas can be

surgically excised, chemotherapy is not the optimal treatment method. Fiber endoscopy can be applied to internal cancers by inserting a catheter intravenously to the area required. Granted, this application extends our horizons, yet it nonetheless limits us in treatment of metastatic proliferation, the most belligerent side effect of invasive cancers.

The Department of Radiology at the Harvard Medical School observed a method to overcome this impediment by taking advantage of the Cerenkov Radiation (CR) effect from radioactive decay, thus moving the light source into the body.[22] When radioactive isotopes decay, they eject charged particles (positrons and electrons), which travel faster than the speed of light in the medium, resulting in emission of UV and visible light. The effect is known as CR and it possesses great potential for photo-induced therapy as it is believed to have limitless tissue penetration (**Figure 13**). Positron Emission Tomography (PET) probes such as 2-deoxy-2-[^{18}F] fluoro-D-glucose (FDG) are ideal candidates for CR due to the high β+ emission of the ^{18}F, as well as its short lifetime. In 110 min, FDG transforms to a regular glucose molecule.

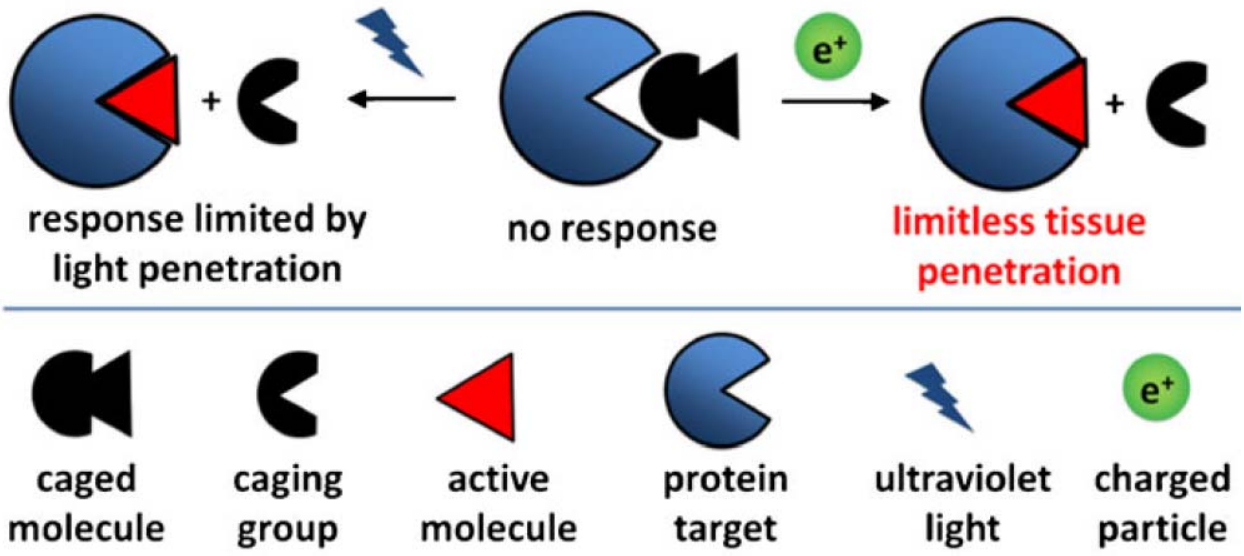


Figure 13: Cerenkov radiation and β+ radioactive isotopes can provide an internalized light source needed to activate an UV dependent prodrug.[22] Figure courtesy of Chongzhao Ran, Zhaoda Zhang, Jacob Hooker and Anna Moore.

For the next step of our project, we propose to use the FDG radionuclide analogously with the Doxaz-MARCKS prodrug in-vivo. Previous studies state that FDG and glucose are not discerned by glucose transporters.[23] In addition, metastatic cancers require higher glucose uptakes due to increased proliferation rates.[23] Based on this evidence, we hypothesized that oncogenic tissues will ingest larger concentrations of FDG. Thus, injection of FDG followed by a waiting period and then the prodrug will supplement the necessary light to cleave the linker at oncogenic (and metastatic) locations as each molecule localizes to the oncogenic sites via its own mechanism.

Conclusion

In this study, we sought to explore synthesis of an improved cancer targeting drug that would have fine selective activation, localization and cytotoxic properties when activated. This was accomplished by incorporating the photolabile linker, which connected the localizing MARCKS peptide, and the Doxaz cytotoxic drug. Throughout we circumvented use of harsh reagents and conditions to prevent anthracycline degradation. Instead, we relied on the facile carbamoylation of the oxazolidine amine, the Cu-free click chemistry and the successive Michael reaction. NMR, ESI-MS and HPLC taken throughout the synthesis verified the structures of the prodrug and its synthetic intermediates. IC_{50} values indicate that the photolabile linker is indeed an improved activation marker. Further studies will be implemented to establish the prodrug's mechanism. Recent research regarding CR in-vivo activation broadens the potential application of Doxaz-MARCKS. We predict that if the radioactive FDG is administered prior to our prodrug, then it can provide the UV light needed to activate the cytotoxic Doxaz selectively at oncogenic tissues. Characteristics of Doxaz-MARCKS and its reported effect in-vitro suggest that in-vivo studies should be pursued, especially in collaboration with CR. Cancer is a hostile and widespread pathology, but with innovative tools better treatments can be employed.

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